I HEREBY CERTIFY THAT THIS CORRESPONDENCE IS BEING DEPOSITED WITH THE UNITED STATES POSTAL SERVICE AS FIRST CLASS MAIL IN AN ENVELOPE ADDRESSED TO: ASSISTANT COMMISSIONER FOR PATENTS, WASHINGTON, D.C. 20231, ON THE DATE INDICATED BELOW.

BY: Martha O. Helen DATE: August 19, 1998

PATENT
BOX NON-FEE AMENDMENT

Attached
Paper #12

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re:	Patent Application of James A. Hoxie	: Group Art Unit: 1648
Appln. No.:	08/882,435	: Examiner: B. Nelson
Filed:	June 25, 1997	:
For:	ANTIBODIES DIRECTED AGAINST CELLULAR CORECEPTORS FOR HUMAN IMMUNODEFICIENCY VIRUS AND METHODS OF USING THE SAME	: Attorney Docket No. **9596-11U1** (I-1470)

DECLARATION UNDER 37 C.F.R. § 1.131

I, James A. Hoxie, hereby declare as follows:

1. I am the inventor of the subject matter claimed in the above-captioned application.

2. The claimed invention was made prior to the publication of Feng et al., 1996, "HIV-1 Entry Cofactor: Functional cDNA Cloning of a Seven-Transmembrane, G Protein-Coupled Receptor," Science 272:872-876, and prior to the publication of Cohen, 1996, "Research News: Likely HIV Cofactor Found," Science 272:809-810. Support for this is found in the accompanying photocopies of original pages from two separate Applications for Continuation of Grant all of which demonstrate identification of the claimed anti-immunodeficiency virus antibody (*i.e.*, 12G5) prior to the publication of Feng et al., *supra*, and Cohen, *supra*, on May 10, 1996. These photocopies form Exhibit A attached hereto. The dates on these photocopies have been redacted, but these pages demonstrate that both Applications were filed with an appropriate government agency prior to May 10, 1996.

3. I declare further that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

8/18/98
Date

James A. Hoxie
James A. Hoxie, M.D.

ARRA

Form approved through OMB No. 09

DEPARTMENT OF HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE

APPLICATION FOR CONTINUATION GRANT

REVIEW GROUP	TYPE ACTIVITY	GRANT NUMBER

TOTAL PROJECT PERIOD
From: Through:

REQUESTED BUDGET PERIOD
From: Through:

To be verified by applicant. Check information in items 1 through 6. If incorrect, furnish correct information in item 13.

1. TITLE OF PROJECT
VIRAL AND CELLULAR DETERMINANTS OF SIV PATHOGENESIS

2a. PRINCIPAL INVESTIGATOR OR PROGRAM DIRECTOR *(Name and address, street, city, state, zip code)*
HOXIE, JAMES A
UNIV OF PENNSYLVANIA
3400 SPRUCE ST
PHILADELPHIA, PA 19104

4. APPLICANT ORGANIZATION *(Name and address, street, city, state, zip code)*
UNIVERSITY OF PENNSYLVANIA
OFC OF RESEARCH ADMINISTRATION
133 SOUTH 36TH STREET, SUITE 300
PHILADELPHIA, PA 19104-3246

BITNET/INTERNET ADDRESS

5. ENTITY IDENTIFICATION NUMBER 1231352685A1

2b. DEPARTMENT, SERVICE, LABORATORY OR EQUIVALENT
MEDICINE

2c. MAJOR SUBDIVISION
SCHOOL OF MEDICINE

3. ORGANIZATIONAL COMPONENT TO RECEIVE CREDIT FOR BIOMEDICAL RESEARCH SUPPORT GRANT *(See Instructions)*
01 SCHOOL OF MEDICINE

6. TITLE AND ADDRESS OF ADMINISTRATIVE OFFICIAL
DIRECTOR
UNIVERSITY OF PENNSYLVANIA
OFC OF RESEARCH ADMINISTRATION
133 SOUTH 36TH STREET, SUITE 300
PHILADELPHIA, PA 19104-3246

BITNET/INTERNET ADDRESS

Complete the following *(see instructions)*

7. HUMAN SUBJECTS — If "YES" exemption no. or IRB approval date — 4b. Assurance of compliance no.
[X] 7a. NO [ ] YES

8. VERTEBRATE ANIMALS — If "YES," IACUC approval date — 8b. Animal welfare assurance no.
[ ] 8a. NO [X] YES A3079-01

9. PERFORMANCE SITE(S) *(Organizations and addresses)*
CLINICAL RESEARCH BUILDING, RM 670
UNIVERSITY OF PENNSYLVANIA
422 CURIE BOULEVARD
PHILADELPHIA PA 19104

10. COSTS REQUESTED FOR NEXT BUDGET PERIOD
10a. DIRECT $ 170,819 10b. TOTAL $278,862

11. INVENTIONS AND PATENTS *(See instructions)*
[X] NO [ ] YES If "YES," [ ] Previously reported [ ] Not previously reported

TELEPHONE AND FAX INFORMATION

	AREA CODE	TELEPHONE NO. AND FAX NO.
12a. PRINCIPAL INVESTIGATOR OR PROGRAM DIRECTOR *(Item 2a)* JAMES A. HOXIE	215	898 0261 (TEL) 662 7617 (FAX)
12b. NAME OF ADMINISTRATIVE OFFICIAL *(Item 6)* Anthony Merritt	215 215	898-7293 898-9708
12c. NAME AND TITLE OF OFFICIAL SIGNING FOR APPLICANT ORGANIZATION *(Item 15)* Berenice N. Saxon, Assistant Director, Research Administration	215	898-7243

BITNET/INTERNET ADDRESS

13. USE THIS SPACE FOR CORRECTIONS TO ITEMS 1 THROUGH 6. INDICATE THE NUMBER(S) WHERE ANSWERS APPLY.

14. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR ASSURANCE: I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application. Willful provision of false information is a criminal offense (U.S. Code, Title 18, Section 1001). I am aware that any false, fictitious, or fraudulent statement may, in addition to other remedies available to the Government, subject me to civil penalties under the Program Fraud Civil Remedies Act of 1986 (45 CFR 79).	SIGNATURE OF PERSON NAMED IN 2a *(In ink. "Per" signature not acceptable.)* James A Hoxie	DATE
15. CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true and complete to the best of my knowledge, and accept the obligation to comply with the Public Health Service terms and conditions if a grant is awarded as the result of this application. A willfully false certification is a criminal offense (U.S. Code, Title 18, Section 1001). I am aware that any false, fictitious, or fraudulent statement may, in addition to other remedies available to the Government, subject me to civil penalties under the Program Fraud Civil Remedies Act of 1986 (45 CFR 79).	SIGNATURE OF PERSON NAMED IN 12c *(In ink. "Per" signature not acceptable.)* Berenice Saxon	DATE

Cys mutation in the TM tail, termed BK/Cys env. This virus was replication competent, and in 2 different cell types, HUT and CEMx174, produced a 4 to 10 fold increase in surface envelope as determined for FACS analysis with monoclonal antibodies and serum from an infected macaque. The amount of envelope on virions as determined by western blot and morphologic analysis by electron microscopy was also increased compared to BK28. This important finding has for the first time identified a single amino acid in the cytoplasmic tail of a retrovirus that can markedly upregulate envelope amounts on infected cells and virions.

Specific Aim #2- Structure/Function analysis of the CP-MAC envelope.

As noted above, a single amino acid change of a Tyr to a Cys in the TM cytoplasmic tail was shown to markedly increase the level of expression of envelope on infected cells and virions. Studies have been undertaken to evaluate the mechanism for this effect. Our preliminary data had indicated that SIV envelope glycoproteins are actively endocytosed, at least in part, through clathrin-coated pits. With the availability of viruses differing only in a Tyr or Cys at the putative endocytosis domain, we were able to address the question of whether or not this change conferred a difference in the rate of endocytosis. Although quantitative data is only preliminary (see below) we have determined using immunoelectron microscopy that the Cys substitution produces a striking redistribution of envelope on infected cells such that the entire plasma membrane is labeled. In contrast, for cells infected by viruses with a Tyr, envelope is located predominantly in patches and in coated pits.

In order to determine if an endocytosis motif existed in the SIV TM tail and to obtain quantitative data on the effect of the Tyr to Cys change on its function, we produced chimeric molecules containing the external and membrane spanning domains of CD4 and the cytoplasmic domain of SIV TMs containing either a Tyr or a Cys at position 723. These constructs have been inserted into retroviral vectors and stably expressed in Hela cells and a CD4 negative variant of Sup-T1 cells derived in our laboratory. Both CD4/SIV-Tyr and CD4/SIV-Cys chimeras are stably expressed on the cell surface, and preliminary results have indicated a 2-4 fold increase in the rate of endocytosis for the Tyr compared to the Cys-tailed construct.

In additional studies we have taken advantage of the high level of surface envelope expressed on the surface of CP-MAC-infected cells to produce a panel of novel monoclonal antibodies raised to native envelope on the cell surface. Mice were immunized with CP-MAC-infected cells and monoclonal antibodies screened for their ability to inhibit cell fusion. We have derived 6 antibodies that are potent inhibitors of cell fusion and which apparently recognize conformational epitopes not involved in CD4 binding. Plans to develop these antibodies for further studies in this Aim are described below.

Specific Aim#3- Evaluation of similar structure function relationships for other HIV and SIV isolates.

With the profound effects of the 723 Tyr to Cys change noted above on surface expression for SIVmac, we have begun to evaluate this determinant for HIV-1. This mutation was introduced into the PNL43 molecular clone of HIV-1 and the resulting virus determined to be replication competent. However, the wild type virus proved to be unable to establish stable long term productive infection and as a result, direct comparisons of Tyr and Cys tailed HIV-1s with regard to surface envelope expression could not be undertaken. Studies are now in progress to introduce this change into other molecular clones of HIV-1, including the macrophage-tropic clone 89.6.

3. Significance.

endocytosis when transferred to a heterologous molecule (CD4). In the coming year we will generate stable cell lines containing CD4 constructs with SIV tails containing a number of different amino acids at position 723 in an attempt to determine their quantitative effect on endocytosis. These results will be compared to the endocytosis rate for the transferrin receptor which is a well characterized, constitutively endocytosed surface protein. In addition we also plan to evaluate endocytosis rates for constructs that contain the membrane spanning domain of SIV as well as the TM cytoplasmic tail to determine if the putative endocytosis motif in the SIV tail can function more efficiently in the context of an SIV MSD. Additional work will attempt to define the various compartments involved in the endocytosis of SIV envelope. In this work we will use anti-lysosome, -Golgi, and -endosomal reagents in double labeling protocols to identify the various pathways involved in the trafficking of endocytosed envelope glycoproteins. We are particularly interested in determining if endocytosed envelope molecules are recycled back to the cell surface. Finally efforts will continue with Dr Timothy Hart at Smith Kline to evaluate the association of cell surface envelope and chimeric CD4/SIV molecules with coated pits, since it is hypothesized that differences in the rate of endocytosis will be reflected in differences in the extent of envelope entry into coated pits.

b. Immunological characterization of CP-MAC envelope. As noted above high envelope expression of CP-MAC virus enabled us to produce a panel of monoclonal antibodies to infected cells that have been extraordinarily potent in their ability to prevent cell to cell fusion. These 6 antibodies will be further characterized as to their mechanism of action, their ability to neutralize virus, and the extent to which they affect infection and fusion for more distantly related members of the HIV/SIV family. Efforts will also be made to determine if these antibodies can be useful in identifying conformational changes in envelope following CD4 binding by FACS analysis and eventually fluorescence energy transfer technology. We will also determine through competition studies the extent to which epitopes are shared by these antibodies. Finally, one antibody not noted above that was determined to be able to block fusion, has actually been shown to be directed to the cell surface rather than to envelope. This antibody, termed 12G5, reacts by western blotting with a 95 kD protein and is clearly not directed at CD4. Efforts will continue to characterize this protein, and to determine by immunoprecipitation if it is a component of an integrin heterodimer since these molecules have been implicated as coreceptors for fusion events involving HIV-1. In addition, preliminary data has indicated that this antibody does not block HIV-1 or HIV-2 mediated fusion, but is a potent inhibitor of SIVmac fusion. Thus it is possible that the 12G5 antibody has identified a novel CD4-associated molecule specifically involved in mediating SIV fusion.

Specific Aim #3- Evaluation of similar structure function relationships for other HIV and SIV isolates.

In the original proposal we indicated that any functional determinants that were identified with our SIVmac model would be evaluated in other HIV and SIV viral systems. As noted above, the identification of a single amino acid change in the SIV TM tail that upregulated envelope expression on cells and virions has raised the possibility that similar effects could be observed for other viruses. A Tyr at SIVmac position 723 is present in the corresponding position of the cytoplasmic tails of all HIV-1, HIV-2 and SIV isolates reported to date. During the coming year, we will evaluate the effects of a Tyr to Cys change in this position for the macrophage tropic clone of HIV-1, 89.6, that is able to establish long term productive infection of CEMx174 cells. Viruses with and with out this mutation will be evaluated with techniques similar to those we have described for CP-MAC including FACS analysis of infected cells, ultrastructural analysis of envelope spikes on virions, and the distribution of surface envelope by immunoelectron microscopy. In addition, both BK28 and CP-MAC, similar to many SIVs propagated in human cells contain a premature stop codon in the TM tail. It remains to be determined if the 723 Tyr to Cys change will have a similar effect on envelope expression for viruses with long cytoplasmic tails. Efforts are in progress to introduce this change into the

DEPARTMENT OF HEALTH AND [redacted]N SERVICES
PUBLIC HEALTH SE[redacted]E

APPLICATION FOR CONTINUATION GRANT

REVIEW GROUP	TYPE	ACTIVITY	GRANT NUMBER
		[redacted]	

TOTAL PROJECT PERIOD
From: Through:

REQUESTED BUDGET PERIOD
From: Through:

To be verified by applicant. Check information in Items 1 through 6. If incorrect, furnish correct information in Item 13.

1. TITLE OF PROJECT
VIRAL AND CELLULAR DETERMINANTS OF SIV PATHOGENESIS

2a. PRINCIPAL INVESTIGATOR OR PROGRAM DIRECTOR *(Name and address, street, city, state, zip code)*
HOXIE, JAMES A
UNIV OF PENNSYLVANIA
3400 SPRUCE ST
PHILADELPHIA, PA 19104-4283

4. APPLICANT ORGANIZATION *(Name and address, street, city, state, zip code)*
TRUSTEES OF THE UNIV OF PENNA
133 SOUTH 36TH STREET, SUITE 300
PHILADELPHIA, PA 19104-3246

BITNET/INTERNET ADDRESS

5. ENTITY IDENTIFICATION NUMBER
1231352685A1

2b. DEPARTMENT, SERVICE, LABORATORY OR EQUIVALENT
MEDICINE

2c. MAJOR SUBDIVISION
SCHOOL OF MEDICINE

3. ORGANIZATIONAL COMPONENT TO RECEIVE CREDIT FOR BIOMEDICAL RESEARCH SUPPORT GRANT *(See instructions)*
01 SCHOOL OF MEDICINE

6. TITLE AND ADDRESS OF ADMINISTRATIVE OFFICIAL
DIRECTOR
UNIVERSITY OF PENNSYLVANIA
133 SOUTH 36TH STREET
SUITE 300
PHILADELPHIA, PA 19104-3246

BITNET/INTERNET ADDRESS

Complete the following *(see instructions)*

7. HUMAN SUBJECTS — If "YES" exemption no. or — IRB approval date — 4b. Assurance of compliance no.
[ ] 7a. NO [X] YES

8. VERTEBRATE ANIMALS — If "YES," IACUC approval date — 8b. Animal welfare assurance no.
[ ] 8a. NO [X] YES — A3079-01

9. PERFORMANCE SITE(S) *(Organizations and addresses)*
Clinical Research Building, Rm 670
University of Pennsylvania
415 Curie Boulevard
Philadelphia, PA 19104

10. COSTS REQUESTED FOR NEXT BUDGET PERIOD
10a. DIRECT $ 177,819 10b. TOTAL $ 290,734

11. INVENTIONS AND PATENTS *(See instructions)*
[X] NO [ ] YES If "YES," [ ] Previously reported [ ] Not previously reported

TELEPHONE AND FAX INFORMATION

	AREA CODE	TELEPHONE NO. AND FAX NO.
12a. PRINCIPAL INVESTIGATOR OR PROGRAM DIRECTOR *(Item 2a)* James A. Hoxie		898-0261 Fax
	215	573-2028 Tel
12b. NAME OF ADMINISTRATIVE OFFICIAL *(Item 6)* Anthony Merritt		898-7293 Tel
	215	898-9708 Fax
12c. NAME AND TITLE OF OFFICIAL SIGNING FOR APPLICANT ORGANIZATION *(Item 15)* SUSAN R. PASSANTE ASSISTANT DIRECTOR RESEARCH ADMINISTRATION	215	898-7293 Tel
	215	898-9708 Fax

BITNET/INTERNET ADDRESS

13. USE THIS SPACE FOR CORRECTIONS TO ITEMS 1 THROUGH 6. INDICATE THE NUMBERS(S) WHERE ANSWERS APPLY.

14. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR ASSURANCE: I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application. Willful provision of false information is a criminal offense (U.S. Code, Title 18, Section 1001). I am aware that any false, fictitious, or fraudulent statement may, in addition to other remedies available to the Government, subject me to civil penalties under the Program Fraud Civil Remedies Act of 1986 (45 CFR 79).

SIGNATURE OF PERSON NAMED IN 2a *(In ink. "Per" signature not acceptable.)* James A. Hoxie — DATE

15. CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true and complete to the best of my knowledge, and accept the obligation to comply with the Public Health Service terms and conditions if a grant is awarded as the result of this application. A willfully false certification is a criminal offense (U.S. Code, Title 18, Section 1001). I am aware that any false, fictitious, or fraudulent statement may, in addition to other remedies available to the Government, subject me to civil penalties under the Program Fraud Civil Remedies Act of 1986 (45 CFR 79).

SIGNATURE OF PERSON NAMED IN 12c *(In ink. "Per" signature not acceptable.)* Susan R. Passante — DATE

element is involved, as we had originally hypothesized (see below). In additional studies we have determined that the Y723C mutation of CP-MAC does not in an of itself, alter tropism when introduced into the BK28 virus, nor does it increase the stability of the SU-TM association, which for CP-MAC was shown to be highly stable. Therefore mutations in the external domains of SU and/or TM account for these effects and will represent the focus of future studies. Finally, by evaluating SIVs that differ only in the presence or absence of the Tyr mutation in the cytoplasmic tail, we have determined that an increase in envelope expression has significant biological effects and produces an increased degree of cell killing and fusion.

Specific Aim #2- Evaluation of mechanisms for the effects of envelope mutations in CP-MAC.

a. Studies of an endocytosis motif in the SIV cytoplasmic tail. As previously summarized, we have hypothesized that the Y723C mutation in the TM cytoplasmic tail of CP-MAC increases env surface expression by disrupting an endocytosis motif that is present in the TM cytoplasmic tail. We described in the last progress report the novel finding that envelope glycoproteins which are not incorporated into budding virions are actively endocytosed through clathrin coated pits, and proposed that a mutation in this Tyr could increase surface expression by decreasing the rate of endocytosis. Consistent with this hypothesis, we noted that in cell types infected by viruses containing a 723Y, envelope glycoproteins were located predominantly in clathrin coated pits and in localized patches on the plasma membrane. In contrast, for cells infected by viruses with the Y723C mutation, env was found to be redistributed over the entire plasma membrane. Efforts during the past year have focused on assays to directly demonstrate the presence of an endocytosis motif in the viral TM, and to quantitate the effects of mutations in this region on endocytosis rates.

Chimeric molecules have been created that contain the external and membrane spanning domain from CD4 and an SIV TM cytoplasmic tail derived from BK28. Constructs have been produced that contain SIV tails with either a Tyr, Cys, Phe, Ala or Iso at the position corresponding to Tyr-723. These CD4/SIV chimeric molecules were stably expressed in Hela cells. Control lines were also produced that contained CD4 without a cytoplasmic tail ("tail-less" CD4) and CD4 with the cytoplasmic tail of the LDL receptor, which contains a well characterized endocytosis motif. As expected endocytosis of the CD4/LDL receptor construct was rapid with approximately 15% of the chimeric molecules internalized per min (over the first 5 minutes) and 85% internalized at 60 minutes. In contrast, the "tail-less" CD4 was internalized at a markedly reduced rate (0.75 % per min; 11.4% in at 60 minutes). Remarkably the chimera containing an SIV tail with the Tyr-723 was rapidly endocytosed (5.9% per min; 49% at 60 min) relative to the "tail-less" CD4 (though not as fast as the CD4/LDLr chimera), while mutation of the Tyr to a Cys, Ala, Iso, Ser, and Phe all reduced endocytosis rates (1-2%/min;18-21% internalized at 60 minutes. These findings have provided conclusive evidence that the SIV cytoplasmic tail contains an endocytosis motif that can be transferred to a heterologous molecule and that this motif is dependent on Tyr-721.

b). Production of monoclonal antibodies to CP-MAC envelope glycoproteins. As described in the previous report, we took advantage of the high level of surface envelope glycoproteins of CP-MAC and used infected cells as an immunogen in mice to produce monoclonal antibodies (mAbs) reactive with envelope molecules. It was hypothesized that by expressing high levels of structurally intact envelope glycoproteins that these cells could be an effective immunogen for generating neutralizing monoclonal antibodies. Seven mAbs were described that all inhibited CP-MAC-induced cell fusion, and studies were planned to characterize these mAbs with regard to their mechanism of action, their ability to neutralize SIV, and their cross-reactivity with other isolates.

Considerable progress has been made in the last year. We have determined that all 7 of these mAbs bind to conformational determinants on gp120 and act at a level following binding of the viral envelope to CD4. Importantly, all mAbs have potent neutralizing activity for BK28-related viruses (CP-MAC, SIVmac251, and 32H) and a subset of these antibodies also exhibit cross-neutralizing activity to heterologous sooty mangabey isolates (B670 and smH4). No activity was observed on HIV-1/IIIB. The biological activity of these mAbs is unique among anti-SIV mAbs described previously.

c). Preliminary identification of a candidate CD4-coreceptor molecule for CP-MAC. A serendipitous but potentially important finding from our attempts to produce mAbs to CP-MAC infected cells was the production of a mAb termed 12G5. Like the anti-gp120 mAbs described above, this mAb inhibits cell fusion

of CP-MAC in coculture assays and neutralizes cell-free infection. However, 12G5 reacts with a 95 kD *cellular* molecule that is clearly not CD4. The 12G5 antigen is widely expressed among human hematopoietic cell lines (T, B and megakaryocytic cells) and PBL, but is not expressed on endothelial cells, Hela cells, rhabdomyosarcoma cells or a murine B cell line. Although this anticellular antibody neutralizes CP-MAC, it does not neutralize other isolates of SIV, suggesting that if the 12G5 does function as a CD4 coreceptor for CP-MAC, it is (at least among viruses analyzed thus far) not required for other SIVs.

As an important extension of these studies, we have derived a model for CD4 independent infection in which the 12G5 antigen may be functioning as an alternate receptor. A CD4 negative variant of Sup-T1 cells (termed BC7) has been derived that is resistant to CP-MAC and HIV-1/IIIB infection. However, this cell line has been shown to be highly susceptible to infection, fusion and killing by a variant of HIV-2/NIHz. The extensive cytopathology of this infection has indicated the highly efficient utilization of an alternate receptor. Remarkably, we have determined that infection of BC7 cells by this HIV-2 can be neutralized by 12G5 (at concentrations of 1.0 - 5.0 µg/ml). Moreover, following infection, expression of the 12G5 antigen is markedly downregulated (80-90%), again consistent with it's role as a possible receptor for this virus.

These studies have implicated a 95 kD cellular molecule as a potential CD4-coreceptor for CP-MAC and as an alternate receptor for HIV-2/NIHz. Plans to identify this molecule and to characterize its contribution to viral pathogenesis in both systems will be described.

Specific Aim #3- Evaluation of similar structure function relationships for other HIV and SIV isolates. As noted above, during the last year we have determined that several mutations of a Tyr to a Cys at the corresponding position in the SIVmac239 TM cytoplasmic tail increases expression of env on the surface of chronically infected cells. These studies have indicated that the effects of this mutation on env expression can be seen in the absence of a premature termination codon, and indicate the feasibility of performing studies with these viruses in non-human primates. Finally, in order to determine if similar effects will be seen for HIV-1, a Tyr -> Cys and Tyr -> Iso mutations have been introduced into the 89.6 macrophage tropic clone of HIV-1, and these viruses will be evaluated the coming year.

3. Significance

As described in last year's report, a central problem in studies of HIV and SIV is in understanding the link between genetic and biological diversity among different viruses. Although differences in viral tropism, infection kinetics and cytopathic potential are well described, the genetic determinants and the mechanisms responsible for these differences are poorly understood. The SIV model we originally described has several advantages for addressing these issues, since viruses were derived from a single molecular clone and exhibited marked differences in their host range, cytopathology and structure. In addition, this system has raised other interesting issues concerning the contribution of the host cell to these effects. From the outset, our focus has been not only to genetically map these determinants but to identify the mechanisms involved. Considerable progress has been made in this regard, and a number of novel findings have already resulted from this work.

First, among the complex biological and structural differences exhibited by our CP-MAC variant of SIVmac, we have determined that a single amino acid change in the cytoplasmic tail, was the principal determinant for its increased expression of envelope glycoproteins on the surface of infected cells. During the last year this mutation has been characterized, and a structural motif, involving a highly conserved aromatic amino acid (Tyr-723) in the SIV tail has been implicated. Mutations of this amino acid in the pathogenic SIVmac239 clone have been shown to produce a similar effect, thus enabling the in vivo consequences of this change to be determined (see below). The viral envelope is highly immunogenic and determinants that regulate (positively or negatively) the expression of envelope molecules on the surface of infected cells could have important implications for the ability of infected cells to survive in an infected host, or in their ability to produce viral particles.

Second, in the original proposal, we suggested that the mechanism for the increased expression of envelope glycoproteins of CP-MAC could result from a decrease in the rate of endocytosis caused by the Tyr mutation in the TM tail. This hypothesis was based on predicted structural similarities between this region and a well characterized endocytosis motif in the cytoplasmic tail of the LDL receptor that has been shown to

mediate the constitutive endocytosis of this molecule through clathrin coated pits. Studies in the last progress report noted that for SIVs which maintain this motif, envelope glycoproteins are located predominantly in coated pits whereas for viruses with a mutation at this position, envelope was redistributed over the entire plasma membrane. As described above, by transferring the SIV TM cytoplasmic tail to a heterologous reporter molecule (CD4), we have provided conclusive evidence that an endocytosis signal does exist in the TM tail and that Tyr-723 plays an essential role. These findings place the TM cytoplasmic tail in a pivotal position in the interface between the virus and cell, since viral glycoproteins can apparently be either incorporated into budding virions, or be actively endocytosed. The cellular and viral factors that regulate this branch point during viral assembly will be subject of future study.

Third, as noted above, we have shown that the increased level of envelope glycoproteins expressed on CP-MAC infected cells are potent immunogens for producing monoclonal antibodies. Current vaccine strategies for HIV and SIV have indicated that recombinant or purified preparations of envelope glycoprotein subunits have not preserved structural determinants that are relevant to a protective immune response. We have therefore taken advantage of the extraordinary increase in surface glycoproteins of CP-MAC infected cells to ask whether this conformationally intact form of envelope glycoproteins could elicit antibodies with increased biological activity. As described above, during the last year we have produced and characterized a series of mAbs reactive with the CP-MAC gp120 that are unique among anti-SIV mAbs described to date, in their ability to block cell to cell fusion of SIV and to potently neutralize genetically diverse SIV isolates. Because we have shown that similar mutations can be created in other SIVs that upregulate env expression, this approach could have broad applicability for producing humoral responses with potent antiviral effects. A full evaluation of the utility of this approach for vaccine-related issues is beyond the scope of this proposal. However, these findings have been included as part of a recently submitted RO1 (AI38225, J.A. Hoxie-PI) where it is hoped that the implications of these findings for vaccine strategies can be fully developed.

Fourth, one of the most important findings of this past year has been the characterization of a cellular molecule that may function as a CD4-coreceptor for SIV and as an alternate receptor for some HIV-2s. There is extensive evidence that cellular molecules in addition to CD4 contribute to viral entry either in conjunction with or independent of CD4. However, few of these molecules have been identified. The 12G5 mAb, as described above, that was produced to CP-MAC infected cells reacts with a 95 kD cellular molecule, and has been shown to inhibit CD4-dependent infection (by CP-MAC) as well as CD4 independent infection (by HIV-2). If these preliminary observations are born out by further study, this would be the first identification of a cellular molecule that can function either as a CD4 co-receptor and an alternate receptor. Efforts in the coming year will focus on the identification of the 12G5 antigen and its interactions with the viral envelope. Although it is clear that the 12G5 antigen is not involved in viral entry of all HIVs and SIVs (ie., it does not inhibit HIV-1/IIIB or SIVmac251 infection), its effects in our SIV and HIV-2 models can be rigorously examined and will likely provide more general insights into the envelope receptor interactions of other SIVs and HIVs.

Finally, as we have emphasized, the advantage of our SIV model is that the relevance of pathogenic determinants in the viral envelope can be evaluated in an animal model. As described below, future efforts are directed towards producing viruses that exhibit high levels of envelope glycoproteins on infected cells and that have a low likelihood of reverting in vivo. Because, at least in mice, CP-MAC infected cells elicited novel and potent humoral immune responses, it is possible that viruses which are designed to produce an increase in surface envelope glycoproteins will generate a qualitatively or quantitatively different immune response that could have important implications for viral persistence.

4. Plans in the coming year.

Specific Aim #1- Molecular characterization of CP-MAC.

Although the Y723C mutation was shown to be the principal determinant for the increased levels of envelope glycoproteins on infected cells, this change did not contribute to alterations in tropism. CP-MAC was derived by long term propagation of SIV/BK28 in Sup-T1 cells and exhibited a marked restriction in its ability to infect this cell type. Among a panel of CD4 positive cells, including HUT-78 and CEMx174, CP-MAC that are highly susceptible to the parental SIV/BK28 virus, CP-MAC was only able to infect Sup-T1. In the coming year, efforts will be directed towards identifying the determinants involved. There are 6 amino